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Pluronic F127 hydrogel characterization and biofabrication in cellularized constructs for tissue engineering applications.

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A new method for printing cellularised scaffolds from thermosensitive hydrogels was here proposed. Pluronic F127 solutions and hydrogels in water-based media (15-40 %w/v) were investigated by rheological analysis and tube inverting test. Pluronic F127 hydrogel with 25%w/v concentration was selected as bioink due to its fast gelation at 37°C (5 min), suitable viscoelastic properties (G'= 16500 Pa at 37°C), pseudoplastic behaviour and fast viscosity recovery after shearing (approximately 5 s). Not cellularised and cellularised (with Balb/3T3 fibroblasts) scaffolds with a 0°/90° pattern were fabricated by additive manufacturing technique. Cells were well distributed along scaffold filaments and cell viability was preserved during printing.

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1. Introduction

Hydrogels are three-dimensional (3D) hydrophilic polymeric networks able to retain large amounts of water or biological fluids, and characterized by soft and rubbery consistence in analogy to living tissues [1,2]. Additional advantages of hydrogels are related to the possibility to include biomolecules such as growth factors within the hydrogel network for controlled release [3] as well as the ability of a few types of hydrogels to be used as injectable systems for cell therapy or drug delivery [3-5]. Depending on the mechanism of gel formation, hydrogels can be classified as: (i) chemical (or permanent) gels if the sol-to-gel transition involves the formation of a chemically crosslinked polymeric network [5,6] and (ii) physical (or reversible) gels if the gel forms through non covalent interactions between the chains. In the case of stimuli-sensitive physical gels, the sol-to-gel transition is triggered by changes in temperature (thermo-sensitive hydrogels), pH (pH-sensitive hydrogels) or analyte concentration (analyte-sensitive hydrogels) [7]. In particular, thermo-sensitive hydrogels are interesting in biomedical applications, since temperature control can be easily achieved [8-10].Two different types of thermo-sensitive hydrogels exist that undergo gelation either by cooling below the upper critical gelation temperature (UCGT) or by heating above the lower critical gelation temperature (LCGT), respectively. Hydrogels with LCGT behavior and sol-to-gel transition at 37°C have gained increasing attention in the biomedical field as carriers for cells, drugs and biomolecules, since they allow encapsulation in mild conditions (temperature ≤ 37°C). Such hydrogels can be easily injected *in situ* in the sol state and undergo gelation at body temperature, thus allowing the complete filling of body cavities and defects before gelation.

In the last decade, both chemical and physical hydrogels have been successfully employed as scaffold-forming materials for cell printing technology [11]. Cell printing is a computer-aided tissue engineering technology based on the layered deposition of cellularised hydrogels to form complex 3D constructs [11-13]. In this work, a new approach was developed for cell printing using thermo-sensitive hydrogels. The proposed method was based on the following sequential steps: (i) the cells were first dispersed into a polymeric solution; (ii) the mixture was poured into the dispenser of an additive-manufacturing printer; (iii) sol-to-gel transition was induced by heating the dispenser to 37°C with the aim to avoid cell sedimentation; (iv) finally, a cellularised scaffold was extruded

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layer-by-layer on a thermostated plate at 37°C, according to a computer-driven design.

Pluronics or Poloxamers are non-toxic FDA approved poly(ethylene oxide)/poly(propylene oxide)/poly(ethylene oxide) (PEO-PPO-PEO) triblock copolymers, which aqueous solutions undergo sol-to-gel transition with increasing the temperature above a LCGT. A variety of Pluronics is available on the market, differing for the molecular weight of the building blocks and the ratio between hydrophobic and hydrophilic units. Therefore, Pluronics allow the preparation of thermosensitive hydrogels with different properties, e.g. in terms of critical gelation concentration (CGC) and gelation time at physiological conditions. Pluronic F127 (F127) gels have been widely investigated in the literature as cell and drug carriers thanks to their low toxicity, reverse thermal gelation, high drug loading capabilities and ability to gel in physiological conditions at relatively low concentrations [14-18]. In addition, they have also been studied for cell printing applications since they are biologically inert towards multiple cell types, gel between 10 and 40°C (depending on the concentration), show a broad range of viscosities and can be easily printed without excessive stress for the encapsulated cells [19-21]. Moreover, they can be easily rinsed away after printing (if desired) by simply decreasing the temperature below the LCGT. In this work, F127-based hydrogels, prepared in deionized water, phosphate buffered saline and Dulbecco's Modified Eagle Medium (with concentrations in the 18-40 % w/v range) were prepared and characterized by rheological analysis, tube inverting and gelation time tests. The aims of this characterization were: (i) to study the effects of solution concentration and solvent type on hydrogel properties, and (ii) to select an optimal F127 concentration for the preparation of cellularised scaffolds by the here developed cell printing approach through additive-manufacturing of cellularised thermosensitive hydrogels.

2. Materials and Methods

2.1. Materials

Pluronic F127 (F127, M_n: 12600 Da, 70%w/w PEO) and all solvents were purchased from Sigma-Aldrich, Italy.

2.2. Hydrogel sample preparation

Hydrogel samples were prepared by dissolving F127 powder at predefined concentrations (%w/v) in an aqueous medium - deionized water, phosphate buffered saline (PBS, pH 7.4) or Dulbecco's Modified Eagle Medium (DMEM) with low glucose content - at 6°C to avoid micellisation and/or gelation during solution preparation.

2.3. Rheological characterization

Rheological tests on F127 solutions in deionized water, PBS or DMEM were performed by using a stress-controlled rheometer (MCR302, Anton Paar GmbH), equipped with 25 mm parallel plates. For temperature control, a Peltier system was employed.

The viscous properties of sol phase were studied at constant temperature by means of flow curves (0°C, shear rate from 1 to 100 s^{-1}). Gel viscosity was estimated by using the same procedure, at 37°C,while the yield stress of the gels was calculated by extrapolation of flow curves at zero shear rate. Samples were poured on the rheometer lower plate at 0°C, heated to 37°C in the case of gel characterization, maintained in quiescent conditions for 15 minutes to reach the thermal stability and, finally, isothermally tested.

The viscoelastic properties of the gel phase were investigated by means of frequency sweep tests in Small Amplitude Oscillatory Shear (SAOS) conditions (frequency range from 0.1 to 100 rad/s, strain=0.1%, 37°C). The morphology and the entanglement spacing were evaluated from the linear viscoelastic response, using the frequency dependence of the elastic modulus [22].

Gel structure recovery was investigated at 37°C by subjecting the hydrogel to 100 s^{-1} flow for 10 s followed by 100 Pa stress for 140 s.

Finally, temperature ramp tests at different heating rates and constant shear rate were carried out to obtain information about sol-to-gel transition (temperature ranging from 0°C to 40°C and shear rate=10 s^{-1}). Temperature ramp rates were: 1, 2.5, 5 and 10°C/min.

Flow curve tests were conducted on F127 solutions with concentrations 18, 20 and 25%w/v, while sol-to-gel transition was investigated for F127 solutions with concentrations of 15, 18, 20, 25 and 30 %w/v. Structure recovery studies were carried out for F127 solutions with a concentration of 20 and 25 %w/v.

2.4. Tube inverting test

Sol-gel-sol phase transition behavior of aqueous Pluronic F127 solutions was investigated using the tube inverting method [23-24].

Each solution at a given concentration ranging between 18 and 40%w/v was prepared following a previously reported protocol. A solution volume of 1.5 mL was put into a Bijoux sample container (Sigma-Aldrich, Italy) with an inner diameter of 17 mm. Each sample was subjected to a controlled temperature increase from 6°C to 80°C, at a rate of 1°C/step. Each step consisted of a 1°C temperature increase, followed by isothermal maintenance for 5 minutes and tube inversion, that allowed a visual inspection of the occurrence of phase transition. The sol and the gel were identified as "flow liquid sol" and "no flow solid gel" in 30s inspection, respectively.

2.5. Gelation time in physiological conditions

The gelation time of F127 solutions at physiological conditions was studied by incubation at 37°C (IKA KS-4000i control) at predefined time intervals (2, 5, 10 minutes), followed by vial inversion. In this case, conditions of sol and gel were defined as "flow liquid sol" and "no flow solid gel" in 60s, respectively.